TGF-β Signaling Is Dynamically Regulated During the Alveolarization of Rodent and Human Lungs

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Although transforming growth factor-beta (TGF-β) signaling negatively regulates branching morphogenesis in early lung development, few studies to date have addressed the role of this family of growth factors during late lung development. We describe here that the expression, tissue localization, and activity of components of the TGF-β signaling machinery are dynamically regulated during late lung development in the mouse and human. Pronounced changes in the expression and localization of the TGF-β receptors Acvrl1, Tgfbr1, Tgfbr2, Tgfbr3, and endoglin, and the intracellular messengers Smad2, Smad3, Smad4, Smad6, and Smad7 were noted as mouse and human lungs progressed through the canalicular, saccular, and alveolar stages of development. TGF-β signaling, assessed by phosphorylation of Smad2, was detected in the vascular and airway smooth muscle, as well as the alveolar and airway epithelium throughout late lung development. These data suggest that active TGF-β signaling is required for normal late lung development. Developmental Dynamics 237:259–269, 2008. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

The process of lung development strives to maximize the gas exchange surface area, while minimizing the blood–air barrier (Roth-Kleiner and Post, 2003; Copland and Post, 2004). This is achieved by branching of developing airways in early lung development and progressive subdivision of developing airspaces into alveoli in late lung development by a process of alveolarization (Roth-Kleiner and Post, 2003; Copland and Post, 2004). Alveolarization begins with branching of distal airway saccules into immature alveoli, in the canalicular phase of lung development, and continues through the saccular phase, into the alveolar phase, where alveolar septae, supported by the extracellular matrix (ECM), divide the terminal respiratory sacculles, increasing the number of alveoli. Alveolarization is driven by growth factor-mediated communication between the different cell types of the developing lung (Warburton et al., 2000; Cardoso, 2001; Jankov and Tanswell, 2004). The pleiotropic roles played by the transforming growth factor-beta (TGF-β) family in control-
ling cell proliferation, transformation and apoptosis, as well as ECM deposition and remodeling, have led to TGF-β being accredited with a key role in lung development (Jankov and Tanswell, 2004; Roth-Kleiner and Post, 2005).

TGF-β signaling is initiated by binding of TGF-β ligands to the type II TGF-β receptor (Tgfr2, also called TβRII), which then complexes with a type I TGF-β receptor (either Tgfr1, also called activin-like kinase [ALK]-5; or Acvrl1, also called ALK-1). The type I receptor transmits signals within the cell by means of second-messenger Smad proteins, namely Smad2, Smad3, and Smad4, or by Smad-independent pathways. TGF-β signaling is potentiated by two accessory type III TGF-β receptors: Tgfr3 (also called betaglycan), and endoglin (CD105, the eng gene-product). TGF-β signaling is also regulated by Smad6 and Smad7, inhibitory Smads that antagonize TGF-β signaling. Activated (phosphorylated) Smads are translocated into the nucleus, where they regulate gene transcription and, hence, cell function (Massagué, 1998).

Several studies have implicated TGF-β signaling in early lung development. All three TGF-β isoforms (Pelton et al., 1991) and type I (Zhao et al., 2000b) and type II (Zhao and Young, 1995) TGF-β receptors are expressed in the embryonic rodent lung. Exogenous addition of TGF-β ligands inhibited airway branching in vitro (Sakurai and Nigam, 1997; Liu et al., 2000), and down-regulation either of Tgfr2 (Zhao et al., 1996), or of Smad2, Smad3, or Smad4 (Zhao et al., 1998), which would block TGF-β signaling, enhanced lung branching in vitro. In line with these findings, overexpression of the inhibitory Smad, Smad7, which antagonizes TGF-β signaling, promoted lung branching in vitro (Zhao et al., 2000a).

TGF-β signaling has received much less attention in the context of late lung development. Adenoviral-mediated transfer of TGF-β1 to the neonatal rat lung (Gauldie et al., 2003) and overexpression of TGF-β1 between postnatal day (P) 7 and P14 in the mouse lung (Vicencio et al., 2004) both disrupted alveolar development, providing indirect evidence that TGF-β was a negative regulator of alveolarization. However, blockade of TGF-β signaling by ablation of Smad3 between days P7 and P28 generated a similar phenotype in mice, indicating that TGF-β can also act as a positive regulator of alveolarization (Chen et al., 2005). Similarly, Smad3 deficiency in mice resulted in progressive airspace enlargement with age, implicating TGF-β in the maintenance of alveolar integrity in the developed lung (Bonniaud et al., 2004). Together, these data suggest that TGF-β plays a finely tuned and key role in the alveolarization process, as well as in the maintenance of alveolar structure. No study to date, however, has assessed the expression of the TGF-β signaling system over the course of late lung development. We describe here that the expression, localization, and activity of components of the TGF-β signaling machinery are dynamically regulated over the course of late lung development in rodents and humans.

RESULTS

Early lung development encompasses the embryonic stage (4–7 weeks after conception in humans, embryonic days [E] 9.5–12 in mice), and continues through the pseudoglandular (5–17 weeks in humans, E12–E16.5 in mice) and canalicular (16–26 weeks in humans, E16.5–E17.5 in mice) stages (Copland and Post, 2004). The alveolarization process begins at the end of the canalicular stage. As late lung development proceeds, distal airways form saccular units in the saccular stage (24–38 weeks in humans, E17.5–P4), and secondary septae then divide these units (septation) during the alveolar stage (36 weeks preterm to 36 months postnatal in humans, P4–P28 in mice; Copland and Post, 2004). A progressive decrease in the size of the alveolar air spaces, together with a concomitant increase in the total number of alveoli, occurs during this critical period of late lung development (Copland and Post, 2004).

The progress of late lung development in the mouse was characterized by dramatic, dynamic changes in the abundance of mRNA encoding components of the TGF-β signaling machinery (Fig. 1) as assessed by semiquantitative and real-time reverse transcriptase-polymerase chain reaction (RT-PCR). In particular, the two type I TGF-β receptors exhibited opposite patterns in mRNA abundance, where acvrl1 mRNA abundance significantly increased over time (compare stages E15 and P21; Fig. 1B,F), whereas tgfbr1 mRNA abundance significantly decreased over time (compare stages E15 and P21; Fig. 1C,F). A biphasic pattern was observed for tgfbr3 mRNA abundance (Fig. 1A,F), while eng mRNA abundance significantly increased during late lung development (compare stages E15 and P21; Fig. 1F). Due to large variations within each group, changes in the abundance of tgfbr2 mRNA were not detected (Fig. 1A,D). The abundance of mRNA encoding Smad proteins was also regulated during late lung development in the mouse. The mRNA levels of smad2, smad3, and smad4 were significantly decreased by day P28 compared with stage E15 (Fig. 1A,G). In the case of smad2, mRNA abundance was significantly reduced as early as day P3 compared with stage E15 (Fig. 1A,G). In contrast, the smad6 and smad7 mRNA levels significantly increased during late lung development in the mouse (compare stages E15 and P21; Fig. 1A,G).

Immunoblot analysis of TGF-β receptors and Smad proteins in extracts from mouse lungs at different stages of development (Fig. 2) revealed comparable patterns to those observed in the mRNA profiles (Fig. 1). Expression levels of Acvrl progressively increased while those of Tgfr1 progressively decreased (compare the expression levels of both molecules at stages E15 and P28 in Fig. 2B). Two splice-isoforms of the tgfbr2 gene have been described (Hirai and Fijita, 1996; Krishnaveni et al., 2006), which encode high and low molecular mass Tgfr2 protein isoforms. Both the higher molecular mass Tgfr2 isoform 1 (also called TβRII-B) and the lower molecular mass Tgfr2 isoform 2 were detected in mouse lung extracts (Fig. 2A,B). Expression levels of Tgfr2 also exhibited dynamic regulation during late lung development, peaking at the late saccular stage (Fig. 2A,B). Consistent with the mRNA abundance patterns, all three Smad proteins investigated, Smad2, Smad3, and Smad4, initially exhibited stable expression.
Fig. 1. Expression of the transforming growth factor-beta (TGF-β) signaling machinery during the late lung development in mice. **A:** Changes in the expression of genes, assessed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR), encoding TGF-β signaling machinery during late lung development. Amplicons are illustrated from two different mouse lungs per developmental stage, representative of the patterns observed in six other mouse lungs at the same developmental stage. **B–E:** Changes in mRNA expression were also confirmed by real-time RT-PCR for the classic TGF-β receptors acvrl1 (B), tgfbr1 (C), tgfbr2 (D), and tgfbr3 (E). **F,G:** Changes in mRNA expression of genes encoding TGF-β receptors (F) and TGF-β Smads (G) assessed by semiquantitative RT-PCR (for which representative examples are illustrated in A) were quantified by densitometric analysis of amplicons from six to eight different mouse lungs per developmental stage. Band intensities (described by pixel density, PD) from samples were normalized for loading using the hspa8 amplicon from the same sample. Data reflect the mean normalized PD ± SD (n = 6–8). *P < 0.05 vs. embryonic day (E) 15. Pseud, pseudoglandular; can, canalicular.
over the course of late lung development; however, the expression of all three Smads was significantly reduced at day P28 compared with stage E15 (Fig. 2A,B). Taking the RT-PCR and immunoblot data together, these data suggest that Tgfr1/Tgfr2/Smad2,3,4-mediated signaling is dampened while Tgfbr1, some staining in the interstitial staining was lost; however, staining was still detected in the airway epithelium and in the vessel walls (Fig. 3F). Additionally, an abundance of cytosolic and nuclear staining was now observed in the epithelial lining of the airways (Fig. 3F).

To localize activated TGF-β Smads, mouse lung sections were stained for phospho-Smad2 (Fig. 4A,B). Nuclear-localized phospho-Smad2 was evident in the interstitium, airway epithelium, and in the vessel walls of mouse lungs at day P7 (Fig. 4A). By day P28, the interstitial staining was lost; however, staining was still detected in the airway epithelium and in the vessel walls (Fig. 4B), indicating that TGF-β signaling was broadly active at the start of alveolarization (day P7), but was confined to the epithelial lining of airways toward the end of alveolarization (day P28). Smad activation was assessed directly by immunoblot for phospho-Smad2 in whole-lung extracts from mice over the course of late lung development (representative blots are illustrated in Fig. 4C; and multiple blots are quantified in Fig. 4E,F). A robust phospho-Smad2 signal was obtained at the early alveolarization stages (days P3 and P7 in Fig. 4C,F), which is consistent with the immunohistochemical data presented in Figure 4A. By day P28, the phospho-Smad2 signal was significantly reduced (day P28 in Fig. 4C,F). However, normalization of the phospho-Smad2 signal for total Smad2 (Fig. 4F) clearly illustrates that, while the abundance of phospho-Smad2 at day P28 is significantly less than that at day P3 (Fig. 4E), the proportion of the total Smad2 pool that is activated (phosphorylated) is significantly increased at day P28 compared with day P7 (Fig. 4F). These data suggest that TGF-β signaling remains active into late lung development and, together with the histochemical data for phospho-Smad2 (Fig. 4A,B), suggest that mice at day P7 (Fig. 3E). An abundance of nuclear-localized staining for Smad3 was observed in this tissue, indicating that TGF-β signaling was active. Little or no staining for Smad3 was observed in the in the airway epithelium (Fig. 3E). By day P28, much of the interstitial staining for Smad3 was lost, although staining, including nuclear staining, for Smad3 was still observed in the vessel walls (Fig. 3F). Additionally, an abundance of cytosolic and nuclear staining was now observed in the epithelial lining of the airways (Fig. 3F).

In addition to changes in total expression levels in the lungs of healthy mice, both TGF-β receptors and Smad proteins exhibited different localization during late lung development. Antibodies directed against Acvrl1 were not suitable for immunohistochemical assessment of Acvrl localization in the mouse (data not shown); however, both Tgfr1 (Fig. 3A) and Tgfr2 (Fig. 3C) exhibited staining in the vessel walls of the developing lung at postnatal day P7, and in the case of Tgfr1, some staining in the interstitium was also observed. In contrast, no staining was observed in the epithelial lining of the airways (insets to Fig. 3A,C). Toward the end of alveolarization (stage P28), staining in vessel walls for Tgfr1 (Fig. 3B) and Tgfr2 (Fig. 3D) was lost, although staining was now evident in the epithelial lining of the airways.

Smad protein localization was also different during late lung development (Fig. 3E,F). Staining for Smad3 was evident in the vessel walls, including the endothelium, as well as in the interstitium and in the subepithelial layer of the airways in the lungs of
the bulk of this signaling at day P28 occurs in the airway epithelium.

TGF-β signaling was also assessed indirectly, in whole-lung extracts from mice over the course of late lung development using two TGF-β/Smad2,3 target genes: *ctgf* (encoding connective tissue growth factor) and *serpine1* (encoding plasminogen activator inhibitor-1; Wu et al., 2007; Zakrzewicz et al., 2007). Whereas both genes were expressed throughout late lung development (Fig. 4D), their expression was significantly increased at day P28 compared with stage E15 (Fig. 4G). These data are consistent with the increased proportion of phospho-Smad2 seen at day P28 (Fig. 4F). However, they are not consistent with the increased overall abundance of phospho-Smad2 between days P3 and P14. It may well be that these genes are induced by TGF-β in specific cell types (for example the airway epithelium) where TGF-β/Smad2,3 signaling is active only later in lung development (as suggested by the immunohistochemical analysis for Smad3 and phospho-Smad2 in Figs. 3 and 4). Alternatively, the expression of *ctgf* and *serpine1* may also be positively and negatively regulated by other factors active during lung development, hence, the lack of correlation with the overall abundance of phospho-Smad2 between days P3 and P14. Indeed, *ctgf* can also be induced by, for example, angiotensin II (Iwanciw et al., 2003) and *serpine1* can be induced by mechanical stimulation of the airways (Chu et al., 2006). Because all of these factors do impact late lung development, *ctgf* and *serpine1* expression may not represent ideal indirect readouts of TGF-β/Smad2,3 signaling in this context.

To assess the extent to which these patterns were comparable to human lung development, sections of healthy human lungs in the canalicular, saccular, and alveolar stages of development were screened by immunohistochemical analysis for the expression and localization of components of the TGF-β signaling machinery (Figs. 5, 6). In whole-lung extracts from mice, one of the type I TGF-β receptors, Acvrl1, exhibited a progressive increase in expression as late lung development proceeded, as assessed by RT-PCR (Fig. 1A,B,F) and immunoblot (Fig. 2A,B). A similar trend was observed in human lungs, where progressively more Acvrl1 staining was observed in the canalicular (Fig. 5A,E), saccular (Fig. 5B,F), and the alveolar (Fig. 5C,G) stages. Diffuse staining was observed in the interstitium throughout these stages. Staining was most evident in the bronchial epithelium, and increased in intensity in the vascular smooth muscle (Fig. 5C,G; thick arrows) in the alveolar stage. The anti-Acvrl1 antibody did not stain mouse tissue (data not shown); therefore, comparisons with Acvrl1 localization in rodents could not be made. In the case of the second type I TGF-β receptor, Tgfr1, no staining was evident in the pseudoglandular stages (not shown); how-
ever, staining of uniform intensity was observed diffusely in the interstitial and was pronounced in the airway epithelium in the canalicular, saccular, and alveolar stages of human development (Fig. 5A–C,E–G). Additionally, pronounced staining was observed in the vascular smooth muscle in the saccular and alveolar stages (Fig. 5B,C,F,G). A similar staining pattern was observed for Tgfrb2 in human lungs (Fig. 6A–C,E–G), where diffuse staining was evident in the interstitium, as well as in the epithelial lining of the developing airways. This staining increased in intensity between the canalicular and alveolar stages, becoming most intense in the alveolar stage (Fig. 6C,G). Staining for Tgfrb2 was also evident in the vessel walls in the saccular (Fig. 6B,F) and alveolar (Fig. 6C,G) stages.

Active TGF-β signaling was demonstrated throughout human lung development by the presence of nuclear...
localized phospho-Smad2 in the developing lungs, in the canalicular (Fig. 6H,L), saccular (Fig. 6L,M), and alveolar (Fig. 6J,N) stages. Staining was observed in the interstitium, in the vessel walls and was pronounced in the epithelial lining of the developing airways (Fig. 6). Additionally, nuclear phospho-Smad2 staining was also observed in the airway smooth muscle in the alveolar stage of human lung development (Fig. 6J,N). Together, these data indicate that components of the TGF-β signaling machinery are expressed during late lung development in humans and that the expression of various components of this machinery are differentially regulated as late lung development proceeds. Additionally, TGF-β signaling per se was active during human late lung development. The alveolar phase of lung development in humans spans the period from 36 weeks gestation to 2.5 years postnatal. The tissue sections illustrated here are derived from neonatal patients who died preterm in utero, or in the first 3 weeks after delivery. Therefore, all sections illustrated here are in the very early alveolar stage of lung development, probably equivalent to between days P3 and P7 in neonatal mouse lungs. With this in mind, the expression patterns observed for Acrvl1, Tgfrbr1, Tgfrbr2, and phospho-Smad2 appear to be consistent between mouse and human over the course of late lung development.

**DISCUSSION**

Our data suggest that TGF-β signaling may be required for normal late lung development in mammals. Some notable patterns in TGF-β receptor expression were observed. Two type I TGF-β receptors exist: Acrvl1 and Tgfrbr1. The expression of these two receptors was differently regulated in the mouse, with a progressive increase in Acrvl1 expression and a progressive decrease in Tgfrbr1 expression during late lung development. Acrvl is believed to play a role in the endothelium in the maturation phase of angiogenesis (Lamouille et al., 2002), while the more ubiquitously expressed Tgfrbr1 mediates ECM deposition and remodeling (Massagué, 1998). The patterns in Acrvl vs. Tgfrbr1 expression that we observed over late lung development suggest that endothelial maturation is favored, while ECM production is gradually down-regulated. However, pronounced staining for Acrvl was also noted in the vascular smooth muscle and the airway epithelium, for which no function has yet been ascribed. To our knowledge, this is the first report of Acrvl1 expression in either cell type.

What other roles may TGF-β play during late lung development? Dynamic changes in the expression of components of the TGF-β signaling system were observed in several compartments of the developing lung. Diffuse staining in the interstitium was observed throughout late lung development. Type II pneumocytes serve as progenitor cells for the alveolar epithelium and are an important component of the interstitium. The differentiation of type II pneumocytes is regulated, at least partially, by a balance between glucocorticoid and TGF-β signaling (McDevitt et al., 2007). Furthermore, transdifferentiation of type II pneumocytes into type I pneumocytes is an important feature of the alveolarization process (Copland and Post, 2004). TGF-β/Smad2,3,4 signaling plays a key role in this type II to type I pneumocyte transdifferentiation (Bhaskaran et al., 2007), which may explain the active TGF-β signaling observed in several compartments of the developing lung. Type II to type I pneumocyte transdifferentiation would occur. The most pronounced staining for TGF-β signaling components in mouse and human tissue was observed in the airway epithelium. Furthermore, pronounced staining for nuclear-localized Smad3, and nuclear-localized phospho-Smad2 was observed in the airway epithelium as late lung development proceeded. TGF-β is a key growth factor driving terminal squamous differentiation of cultured bronchial epithelial cells in vitro, as well as changes in airway epithelial cell shape, surface area, and adhesion properties (Masui et al., 1986). Those data suggest that TGF-β is...
critically involved in airway development, which is consistent with the staining patterns that we observed in the airway epithelium during late lung development. In support of this idea, a significant reduction in the abundance of *tgfb1* (encoding the TGF-β1 ligand) and *tgfbr1* mRNA, as well as a significant reduction in the protein expression of TGF-β1 and TGF-β2 ligands, and Tgfr1 and Tgfbr2, have been reported in the bronchial epithelium and smooth muscle of a patient with pulmonary acinar aplasia, a severe form of pulmonary hypoplasia (Chen et al., 1999).

Detection of phospho-Smad2 in airway smooth muscle indicated that TGF-β signaling was also active in this tissue. TGF-β exhibits pro-proliferative effects on airway smooth muscle cells (Xie et al., 2007), and active TGF-β signaling in the airway smooth muscle in the developing lung may contribute to the muscularization of the developing airways during late lung development. This idea is supported by studies in asthma, where overactive TGF-β signaling is believed to underlie the airway smooth muscle cell hyperplasia observed in asthmatic patients (Xie et al., 2007).

While TGF-β signaling is required for normal late lung development, this requirement is clearly finely tuned, because too much TGF-β signaling can negatively impact alveolarization. Neonatal rodents chronically exposed to normobaric hypoxia (Vicencio et al., 2002) or hyperoxia (Alejandre-Alcázar et al., 2007a) exhibited arrested alveolarization and developed bronchopulmonary dysplasia. In the case of hyperoxia, this occurred concomitantly with up-regulated Tgfbr2 and Smad4 expression, and increased TGF-β/Smad2,3 signaling in the developing lung (Alejandre-Alcázar et al., 2007a). Dampening of TGF-β signaling in this model by administration of a neutralizing anti–TGF-β antibody largely restored normal alveolar architecture (Nakanishi et al., 2007), validating a pathological role for TGF-β in hyper-

**Fig. 6.** Changes in the expression levels and localization of the type II transforming growth factor-beta (TGF-β) receptor and phospho-Smad2 during human late lung development. **A–N:** The type II TGF-β receptor Tgfbr2 (A–G) and phospho-Smad2 (H–N) were localized in the developing lungs of patients at the canalicular, saccular and alveolar stages, by immunohistochemistry. D and K illustrate staining of sections where the immune antibody was preadsorbed with competing peptides for Tgfbr2 (D) or phospho-Smad2/3 (K). The areas illustrated are representative of staining patterns observed in three to five different patients per developmental stage. Where high-magnification images (lower row) are derived from the low-magnification image (upper row), the magnified area is demarcated with a solid-lined box. Thin arrows indicate examples of staining in cells lining conducting airways and in the interstitium. Thick arrows indicate examples of staining in vessel walls. aw, airway; v, vessel.
oxia-induced arrest of alveolar development. At the same time, complete blockade of TGF-β signaling by ablation of Smad3 in neonatal mice also arrested alveolarization (Chen et al., 2005). Combining the ideas in those reports with data presented here, it is emerging that, although TGF-β is a negative regulator of airway branching in early lung development, we demonstrate here that TGF-β signaling is active in multiple tissue types in the lung during normal late lung development. The degree of TGF-β signaling must, however, be precisely controlled, because both up- and down-regulation of TGF-β signaling impairs the alveolarization process.

**EXPERIMENTAL PROCEDURES**

**Animals and Tissue Treatment**

The animal ethics authority of the government of the State of Hessen approved all animal procedures. C57BL/6J mice were housed in humidity- and temperature-controlled rooms on a 12:12-hr light–dark cycle and were allowed food and water ad libitum. Mice at stages E15, E17, E19, P1, P3, P5, P7, P14, P21, and P28 were killed by intraperitoneal injection of sodium pentobarbital. Lungs were excised, flushed free of blood with phosphate-buffered saline (PBS), and processed immediately for RNA or protein extraction. For histology, the heart and lungs from mice at days P7 and P28 were excised on bloc, and the lungs were pressure fixed overnight at 20 cm H₂O with 4% (m/v) paraformaldehyde in PBS (20 mM Tris-Cl, 137 mM NaCl, pH 7.6), as described previously (Alejandre-Alcazar et al., 2007a). Paraffin sections (3 μm) were mounted on poly-L-lysine-coated glass slides, dewaxed with xylene (3 × 5 min), and rehydrated in a graduated series of ethanol solutions (100%, 95%, 70% [v/v], and PBS).

**Human Tissue**

Fetal and neonatal human lung tissue was retrieved from archived autopsy material at the Erasmus University Medical Centre and the University of Giessen Lung Center. Lung samples were normal for their gestational age. All human material was used with the approval of the Human Subjects Review Committees of the Erasmus University Medical Centre and the University of Giessen Lung Center. Autopsy material was derived from fetuses from induced or spontaneous abortions, stillbirth, or neonates that died from nonpulmonary causes within 3 weeks after delivery. None of these infants had infections at the time of death. Tissue was fixed in 4% (m/v) paraformaldehyde in PBS, between 1 and 12 hr after death. Before fixation, tissue was stored at 4°C.

**Immunohistochemistry**

Hematoxylin staining, and expression of TGF-β receptors, Smads, and smooth muscle actin (SMA) was assessed on 3 μm tissue sections as described previously (Morty et al., 2007), with anti-SMA (1:850; clone 1A4; Sigma, Taufkirchen, Germany); goat anti-Acrvl1 (catalog no. AF370; R&D Systems, Wiesbaden, Germany), rabbit anti-Tgfbr1 (designated R-20, catalog no. SC-399; Santa Cruz, San Francisco, CA; 1:50; used with blocking peptide SC-399P), rabbit anti-Tgfbr2 (designated C-16, catalog no. SC-220; Santa Cruz, San Francisco, CA; 1:50; used with blocking peptide SC-220P), rabbit anti-phospho-Smad2 (Ser465/467) (catalog no. 05-953), and mouse anti-Smad3 (clone 2C12; catalog no. MAB10075) were both from Upstate (Charlottesville, VA; both used at 1:50). The anti–phospho-Smad2(Ser465/467) antibody was preadsorbed with a phospho-Smad2/3 competing peptide from Santa Cruz (catalog no. SC-11769P; Santa Fransisco, CA). For competing peptides, antibodies were preadsorbed for 30 min at a ratio of 1:5 (antibody:peptide; mol:mol) before use. No competing peptides were available for the anti-Acrvl1 and anti-Smad3 antibodies. Therefore, in the case of these two antibodies, species-matched isotype control antibodies (available with the Histostain Plus Kit from Zymed, San Fransisco, CA) replaced the primary antibodies. Immune complexes were visualized with a Histostain Plus Kit (Zymed, San Fransisco, CA). Although the specificity of all antibodies used for immunohistochemistry has been validated (Zakrzewicz et al., 2007), additional data are included in the relevant panels using antibodies preadsorbed with a competing peptide, or where primary antibodies were replaced with a species-matched isotype control antibody at the same concentration. For mouse and human tissue, all panels illustrated at a particular developmental stage are derived from the same tissue block. The staining patterns depicted are representative for three mice at the same developmental stage, or for between three and five patients at the same developmental stage.

**RNA Isolation, Semiquantitative RT-PCR and Quantitative Real-Time RT-PCR**

Total RNA was isolated from fresh lung tissue using a Qiagen RNeasy kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Mannheim, Germany). One microliter of the RT reaction served as a template in a PCR reaction using Platinum Taq (Invitrogen, Karlsruhe, Germany). Total RNA was screened by semiquantitative RT-PCR using the following forward (for) and reverse (rev) primers at the stated cycle number: acvr1l (29 cycles): 5′-AGG GCC GAC ATG ATG TGT AGG TG-3′ (for), 5′-GCC GGT TAG GGA TGG TGG GTG TC-3′ (rev); ctgf (29 cycles): 5′-GCC ACC AAG CCG AAG ATT-3′ (for), 5′-AGG CCG CTC TTC TTC TCC A-3′; (rev); eng (22 cycles): 5′-GAG TCC GCT GTG ATC AGC CTG TGG TG-3′ (for), 5′-CCA CAA CAC GAA ATG ACC-3′ (rev); hspa8 (24 cycles): 5′-CAA GCA GCA GAA GCA GAA AGA CAT-3′ (for), 5′-ATA CAC AGC GAA AGA GGA GTG ACA TC-3′ (rev); serpine1 (24 cycles): 5′-CAA CAA GCA GAA CAT-3′ (for), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev), 5′-GAC AGG GTC CAT ACC TAC TCC TGG TG-3′ (rev).
GAG AAC ATG GGA TGG AC-3’ (for), 5’-AGT AGC TGG CTG AGC AGT AA-3’ (rev); smad6 (24 cycles): 5’-GAG CAC CCC CAT CTG CTA CAA-3’ (for), 5’-AAC AGG GGC AGG AGG TGA TG-3’ (rev); smad7 (25 cycles): 5’-GAG GCC GTG TGG CCA AG-3’ (for), 5’-AGC CAC GAG TGT AGC CAT CTT CGT CAA-3’ (for), 5’-AAC AGG GGC AGG AGG TGA TG-3’ (rev); smad7 (25 cycles): 5’-AGA GCG TTC ATG GTT AGG AGG GCG AGG AGT AAA GG-3’ (for), 5’-GGA CGG GAG CGC CTG CC-3’ (rev); tgfbr1 (27 cycles): 5’-AGA GCG TTC ATG GTT AGG AGG GCG AGG AGT AAA GG-3’ (for), 5’-GGA CGG GAG CGC CTG CC-3’ (rev); tgfbr2 (25 cycles): 5’-GAG GCC GTG AGG GCG AGG AGG TGA TG-3’ (rev); tgfbr3 (27 cycles): 5’-GCC CCT CCT TAC TCC AGA AAC AGG GGC AGG AGG GCG AGG AGG TGA TG-3’ (rev); and tgfbr3 (25 cycles): 5’-GCC CCT CCT TAC TCC AGA AAC AGG GGC AGG AGG GCG AGG AGG TGA TG-3’ (rev). Quantitative changes in gene expression were analyzed by comparing the ΔCt values of the genes of interest (using RNA samples from three different animals per developmental stage, each sample assessed in duplicate), normalized for the ubiquitously expressed pseudogene-free hydroxyethylbilane synthase (hmbs) gene.

Protein Isolation and Immunoblotting

Protein extraction from mouse lungs, gel electrophoresis, and immunoblotting were performed as described previously (Alejandre-Alcázar et al., 2007a). Lung protein extracts (10–25 μg) were resolved on 12% polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting. Blots were probed with goat anti-Acryl1 (catalog no. AF370; R&D Systems, Wiesbaden, Germany; 1:1,000), mouse anti-Tgfbr1 (designated R-20, catalog no. SC-399; Santa Cruz, San Francisco, CA; 1:1,500 for mouse), mouse anti-Tgfbr2 (designated C-16, catalog no. SC-220; Santa Cruz, San Francisco, CA), rabbit anti-phospho-Smad2(Ser465/467) (catalog no. 05-953), and mouse anti-Smad3 (clone 2C12; catalog no. MAB10075) were both from Upstate (Charlottesville, VA; both used at 1:1,000), rabbit anti-Smad4 (catalog no. H-552; Santa Cruz, San Francisco, CA; 1:1,000), and rabbit anti-Smad2 (catalog no. 51-1300; Zymed, San Francisco, CA; 1:1,000), while mouse anti–α-tubulin (designated B-7, catalog no. SC-5286; Santa Cruz, San Francisco, CA; 1:2,500) served as a loading control. Peroxidase-conjugated anti-mouse (1:1,000–1:2,000) and anti-rabbit (1:2,000–1:2,500) secondary antibodies were from R&D Systems (Wiesbaden, Germany). The specificity of all antibodies used for immunoblotting has been validated (Alejandre-Alcázar et al., 2007a). Densitometric analysis of bands was performed as described for PCR amplicons, above.

Statistical Treatment of Data

Data are presented as mean ± SD. Differences between groups were analyzed by analysis of variance and the Student-Newman-Keuls post hoc test for multiple comparisons, with a P value < 0.05 regarded as significant.

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